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Biotechnology in Energy Forestry: IEA Symposium Abstracts

W.M. Cheliak and A.C. Yapa, editors

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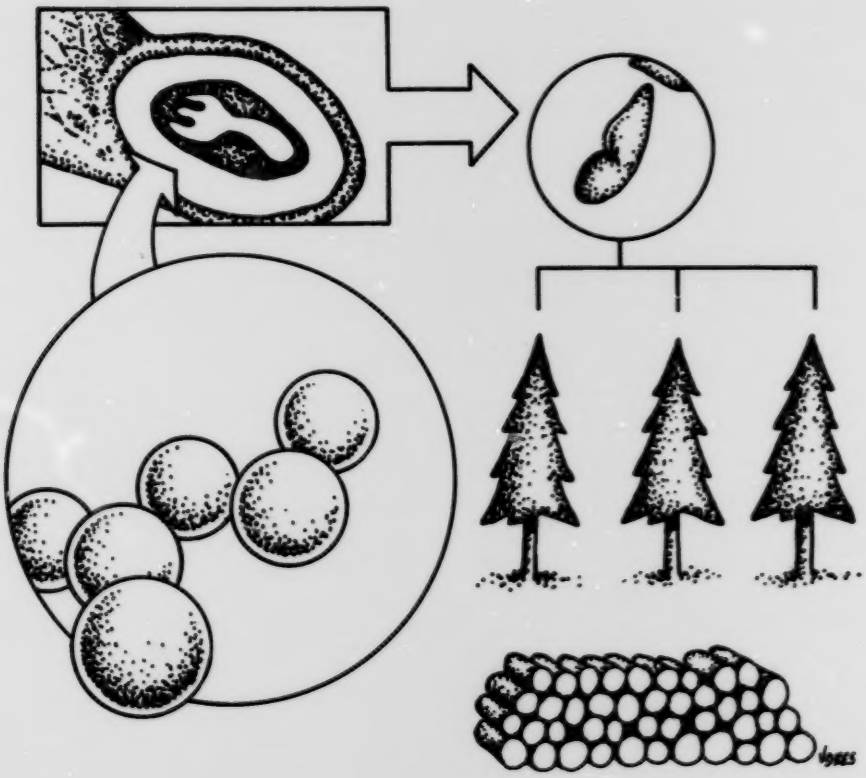

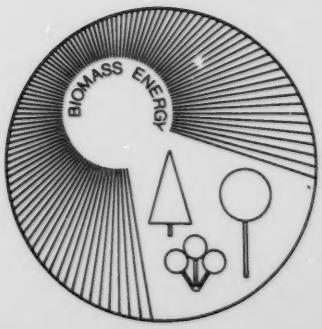

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In common with the rest of the Canadian Forestry Service, the Petawawa National Forestry Institute has as its objective the promotion of better management and wiser use of Canada's forest resource to the economic and social benefit of all Canadians. Objectives of program activities carried out at the Institute support this goal through discovery, development, demonstration, implementation, and transfer of innovations. Because it is a national institute, particular emphasis is placed on problems that transcend regional boundaries or that require special expertise and equipment that cannot be duplicated in CFS regional establishments. Such research is often performed in close cooperation with staff of the regional centres, provincial forest services, and the forest industry.

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FOREST GENETICS AND BIOTECHNOLOGY — Integrates projects in tree genetics, soil microbiology, micropropagation, molecular genetics, meteorology, and seed research. It also includes the client services and seed bank operations of the National Tree Seed Centre, a long-standing program with extensive international affiliations.

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THE RESEARCH FOREST — Besides natural stands manipulated in a variety of ways for silvicultural research, the 98 km^2 Petawawa Forest contains extensive areas of plantations dating back six decades. Research plantations are a source of growth and yield data derived from cultural experiments, and they are becoming valuable for pedigreed genetic materials for micropropagation and molecular genetics studies. The forest also offers opportunities for short- and long-term testing of forest management strategies.

BIOTECHNOLOGY IN ENERGY FORESTRY: IEA SYMPOSIUM ABSTRACTS

W.M. Cheliak and A.C. Yapa, editors

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The symposium was held under the auspices of the International Energy Agency's Bioenergy Agreement Task II.

PREFACE

Biotechnology has caused considerable excitement in numerous areas of biology. In forestry, there are many opportunities where this technology could impact both in upstream (i.e. plant production, growth, and protection) and downstream (i.e. biological conversion and waste management) aspects of activity.

Energy forestry, where crop management is typically more intense than in conventional forestry, provides an unique situation for possible implementation of biotechnological advances. It was with this spirit that this meeting was convened in Uppsala.

Through this meeting we tried to develop biotechnology as a tool to apply to the management of the crop. Thus, there was consideration of germplasm resources, development of genetic resources, crop management, tissue culture, molecular genetics, and genetic engineering. Importantly, there was time for considerable discussion, about details and the possibilities for integration of techniques into a crop management program.

CONFERENCE AGENDA

Tuesday 4 November, 1986

THE CROP: MANAGEMENT, CONVENTIONAL GENETICS AND FUTURE TREES

0830–0900
Welcome and Overview of ESO & IEA
Louis Zsuffa, University of Toronto, CANADA

0900–1000
Germplasm and Conventional Genetics Programs
Veikko Koski and Anneli Viherä-Aarnio
Finnish Forest Research Institute, FINLAND

1000–1100
Break and Posters

1100–1200
Management and Silviculture of Energy Forests
Kurth Perttu, Swedish Agricultural University, SWEDEN

1200–1400
Lunch

1400–1500
Studying Gene Action using Molecular Genetics
Francis Yeh, University of Alberta, CANADA

1500–1530
Break and Posters

1530–1630
Genetic Improvement of Forest Yields – The Future Tree
Warren Nance, USDA Forest Service, USA

Wednesday 5 November, 1986

CELL AND TISSUE CULTURE OF WOODY PERENNIALS

0900–1000
Tissue Culture: Techniques and General Considerations
Tage Ericksson, Institute of Physiological Botany, SWEDEN

0100–1100
Break and Posters

1100–1200
Micropropagation and Organogenesis
Chris Bornman, Hilleshog Research AB, SWEDEN

1200–1400
Lunch

1400–1500
Somatic Embryogenesis
Steve Wann, Institute of Paper Chemistry, USA

1500–1530
Break and Posters

1530–1630
Cell and Protoplast Technology
Brent McCown, University of Wisconsin, USA

Thursday 6 November, 1986

MOLECULAR BIOLOGY AND GENETICS

0830–0930
In vitro disease screening
Frederick Valentine, State University of New York, USA

0930–1030
Molecular Aspects of Plant Development
Ned Kirby, Rutgers University, USA

1030–1100
Break and Poste

1100–1200
DNA Transfer in Pines
Ron Sederoff, USDA Forest Service, USA

1200–
Lunch and tours

1800–
Banquet

Friday 7 November, 1986

GENETIC ENGINEERING

0830–0930
Overview of Gene Transfer Methods
Milt Gordon, University of Washington, USA

0930–1030
Gene Expression in Plants
Tom Parsons, University of Washington, USA

1030–1100
Break and Posters

1100–1200
Development of Glyphosate-tolerant Poplars
Joanne Fillatti, Calgene Inc., USA

1200–1400
Lunch

1400–
Review, discussion, and forward planning

GERMPLASM AND CONVENTIONAL GENETIC PROGRAMS

V. Koski and A. Viherä-Aarnio

Finnish Forest Research Institute
Department of Forest Genetics
Box18, 01301 Vantaa, Finland

The idea of using fast-growing woody plants to produce energy and biomass has become established in several countries (Zsuffa and Barkley 1985). Plantations have been established and harvested on an experimental scale. Even though high yields and large annual increments have been reported from small- sized sample plots, extensive plantations, such as those of the North Carolina cooperative tree improvement program (Weir 1986), are still lacking.

The germplasm used for short rotation experiments consists of land races or wild types of the species in question, rather than products of intentional breeding. This is undoubtedly one reason why the results of experiments have not been encouraging in all cases, particularly when economic aspects are taken into consideration (Hakkila 1985). Successful and profitable short-rotation forestry presupposes both genetically-improved special material and intensive management.

In genetic improvement we have to be ambitious. It is not enough to select superior genotypes or clones from natural populations or from spontaneous hybrids. A more fruitful approach is to design a model plant - ideotype and then, by means of plant breeding, synthesize the corresponding genotype. Desirable components of an ideotype have been suggested by many authors (Dickman 1975, 1985, Siren et al. 1979, Fege 1981, Siren 1985). Here is a partial list:

1. High rate of net photosynthesis
2. Efficient light interception
3. Full utilization of the growing season
4. High harvest index
5. Efficient use of water and nutrients
6. Rapid juvenile growth
7. Tolerance to competition
8. Tolerance to abiotic stress
9. Freedom from pests and diseases
10. Suitable wood properties
11. Ease of reproduction and plantation establishment
12. Affinity to coppicing

It is self-evident that there is no universal ideotype that fits all sites and purposes; each case needs to be tailored separately. The development of synthetic cultivars, in other words the realization of an ideotype, is not possible in one step. All plant breeding is the alternation of selection and recombination. In conventional tree breeding this usually means alternation of progeny testing and crossing. As far as short rotation species are concerned, other more radical methods can be applied as well, such as:

- natural or induced polyploidy,
- hybridization between provenances and species, and
- induced mutations and genetic engineering.

From the palaeobotanic point of view conifers are ancient species whose macroevolution came to a standstill millions of years ago. Their chromosome numbers, for instance, are quite uniform. Deciduous woody plants are considerably younger phylogenetically and contain a diversity of phenotypes. Thus, broad-leaved species are much more plastic.

Within the genera *Betula* and *Salix*, for instance, various levels of polyploidy occur among, and also within, species. For plant systematics these genera are confusing because interspecific hybrids are common, and all species display tremendous morphological variation. Ongoing evolutionary processes, in this case the formation of a new species, have been discovered in *Betula* in Finnish Lapland (Vaarama and Valanne 1973). This suggests that the same kind of potential might also be found in *Salix* and other genera.

The discussion in this paper is limited to species and genera growing in northern temperate climates. Short rotation forestry trials have been established using deciduous species. Most of them are so-called pioneer species and are often undesirable additions in normal forests. Some of the most commonly used genera and species, as well as some of their genetic features, are briefly reviewed next.

From the data available so far, the most promising genera for short rotation forestry appear to be *Alnus, Platanus, Populus,* and *Salix*. The two latter genera have been used more extensively and for a longer period than any others. However, many other genera have potential in short rotation forestry, e.g. *Acer* and *Betula* spp. (Fege 1981, Lavoie and Vallee 1981).

The genus *Alnus* (alder) consists of c.30 species, of which *A. cordata, A. glutinosa, A. incana,* and *A. rubra* are the most economically important (Krüssmann 1976). The natural distribution of genus *Alnus* is presented in Figure 1. Being pioneer species, alders have rapid juvenile growth and take only a short time to reach maturity. Their capacity to fix nitrogen through symbiotic *Frankia* actinomycetes gives alders additional value.

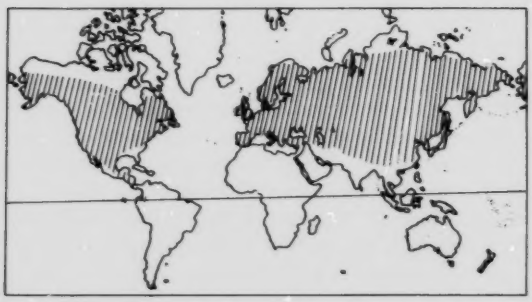

Figure 1. Distribution of genus *Alnus* (According to Krüssmann 1976)

Large genetic variation has been found both among and within alder populations (Debell and Wilson 1978). Interspecific hybrids between sympatric species, e.g. *A. glutinosa* x *A. incana,* occur at a low frequency. Artificial hybrids such as *A. glutinosa* x *A. rubra* have shown good performance (Ljunger 1959, 1972). Alder regenerates vegetatively in nature, and coppicing can be applied successfully.

The genus *Populus* consists of about 35 principal species divided into five sections (Rehder 1947, Krüssmann 1976). The normal chromosome number is 2n = 38, but outstanding polyploid examples have been found in several species. Poplars are predominantly dioecious, but bisexual and hermaphrodite individuals occur frequently. Vegetative regeneration is very vigorous. Interspecific hybridization is common. The genus *Populus* is widely distributed in the northern hemisphere (Figure 2).

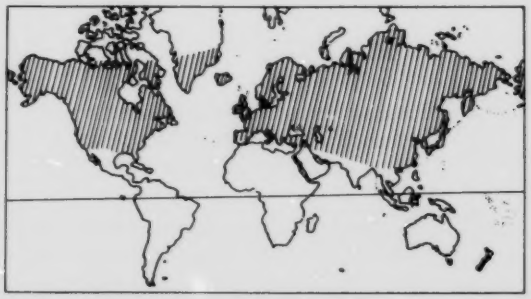

Figure 2. Distribution of genus *Betula* (According to Krüssmann 1976)

Section *Leuce* (aspens) of the genus *Populus* is distributed throughout the northern temperate zone. The main species are *P. tremula* and *P. alba* in Eurasia, and *P. tremuloides* and *P. grandidentata* in North America. Aspens cannot be propagated by rooted cuttings. The section *Aigeiros* contains more than 90% of all cultivated poplars in the world. The two main species are *P. nigra* from the Mediterranean and *P. deltoides* from North America. Cultivars are hybrids, propagated easily from cuttings. The three other sections, *Turanga, Tacamahaca,* and *Leucoides* include interesting species with potential (FAO 1979, Herpka 1985).

As a result of geographic isolation natural hybrids have been heretofore uncommon. Geographic transfers and wide-scale cultivation by humans have broken this geographic isolation and spontaneous hybrids are now common. Interspecific incompatibility is obviously rare in the genus *Populus*. Artificial crossing between species, even between sections, has been successful, and results in large diversity in the offspring (Mühle-Larsen 1970, FAO 1979). Breeding strategies for poplars, especially in Europe, are in a more advanced state than the breeding programs for other species (Teissier de Cros 1984).

The genus *Salix* consists of more than 300 species, subdivided into three subgenera, *Salix, Vetrix,* and *Chamaetia* (Skvortsov 1968). Only species from subgenus *Salix* and *Vetrix* are, however, of interest in biomass production.

The basic chromosome number of *Salix* is x = 19. A whole array of chromosome numbers from 2n = 38 through 2n = 190 has been encountered in *Salix* (Chmelar and Meusel 1976). The main differences in polyploidy are between species, but many species have several levels of polyploidy. Many species are highly variable and hybrids are common. The growth habit varies from average sized trees (*S. alba, S. fragilis*) to dwarf shrubs (*S. herbacea*). Willows are dioecious and insect pollinated. Propagation with cuttings is easy, and the sprouting potential high.

Salix species have an extensive distribution (Figure 3). There are adaptations to various edaphic conditions and climates. Interspecific hybridization is easy. In *Salix* there is a huge gene pool that so far has hardly been utilized (Malmivaara et al. 1971). The few species and origins dealt with are just a small random sample. Systematic work within this natural resource provides an excellent opportunity for genetic improvement.

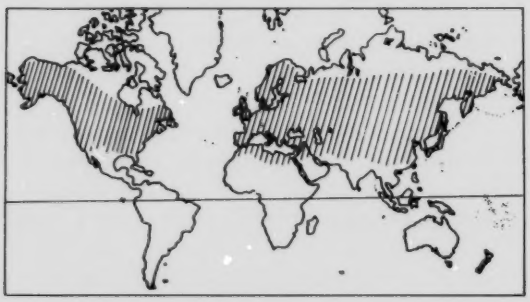

Figure 3. Distribution of genus *Populus* (According to Krüssmann 1976)

The genus *Betula* is distributed over the northern temperate zone (Figure 4). The genus comprises about 50 species, subdivided into three sections; *Nanae, Albae,* and *Costatae.* Birch has, at least in the Scandinavia, some value as a forest tree. Birch has rapid juveline growth and an affinity for sprouting. *B. pendula* can now be propagated vegetatively (Ryynänen and Ryynänen 1986). Birch wood has a high energy value per volume unit (Ferm et al. 1985). The genus *Betula* is genetically highly variable and includes various levels of polyploidy.

What has been discussed so far points to the enormous genetic variability available.

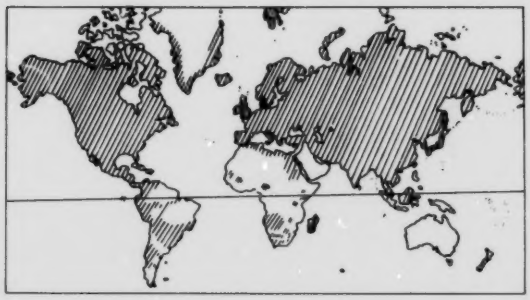

Figure 4. Distribution of genus *Salix* (According to Krüssmann 1976)

However, this wealth is for the most part unexploited. If short rotation forestry for biomass and energy production is to be taken seriously, ambitious and aggressive breeding programs must be carried out. Definition of the goal - the special ideotype - and establishment of breeding populations are the basic starting points. If these steps are followed by selection and recombination cycles, these projects will in a short time provide clones and cultivars which are more productive than those used earlier.

The scenario given by Gullberg and Kang (1985) for a breeding strategy is useful for our purposes (Figure 5). A physically separate, long term breeding population is the substrate of actual genetic improvement. Short term projects apply the results, obtained at intervals, into practice. Supportive research is a catalyst in all activities. As mentioned earlier, genetic improvement is the alternation of selection and recombination. For practical applications, we must bear in mind that superior genotypes must be propagated in sufficient quantities and at a reasonable cost.

Recombination as such, e.g. artificial hybridization of two species with protoplast fusion, is not enough for breeding. Recombinants must appear in large numbers so that selection is possible among them. Desirable combinations appear only at a low frequency.

Biotechnology is indispensable in obtaining the most efficient energy conversions, especially in the following areas:

1. Vegetative propagation of valuable genotypes that cannot be reproduced

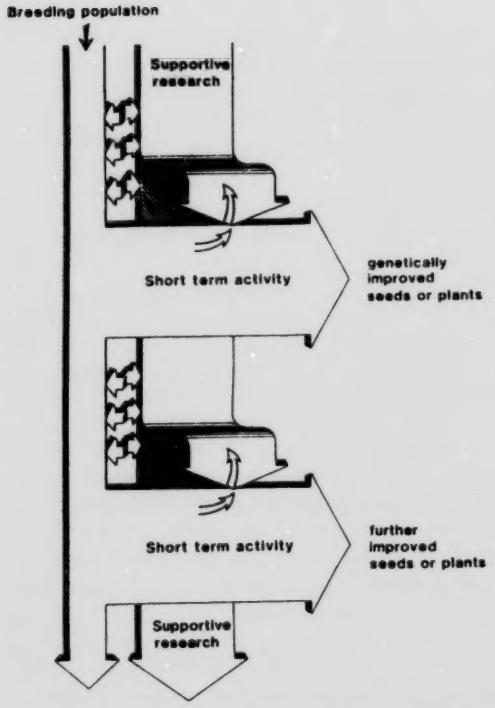

Figure 5. A model for tree breeding (Gullberg & Kang 1985)

sexually. Note that vegetative propagation, e.g. tissue culture, is not the source of the superiority.

2. Recombination provides a greater number of alternations than controlled pollination. The time between generations can be shorter, incompatibility mechanisms between species can be overcome, etc.
3. Microbiological conversion of biomass into sugars, ethanol, methane, amino acids; in other words into more easily transportable forms of raw material.

The progress of biotechnology has been rapid. Recently it has been successfully applied to many forest tree species. For the time being its main role within tree breeding is in supportive research. Because it demands high manual skills biotechnology is not a reasonable method of cloning unselected trees or producing hybrids at random. However, when applied in carefully planned research projects, biotechnology brings a substantial contribution both to long-term breeding populations and short-time projects in producing genetically improved material.

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BIOCHEMICAL AND ULTRASTRUCTURAL CHARACTERIZATION OF EMBRYOGENIC AND NONEMBRYOGENIC CALLUS OF NORWAY SPRUCE

S.R. Wann

Industrial Research Fellow
Union Camp Corporation
c/o The Institute of Paper Chemistry
P.O. Box 1039
Appleton, WI 54912
USA

Introduction

Success in obtaining sustained morphogenesis from cell cultures is often dependent on the ability to recognize, segregate, and selectively proliferate a morphogenic phenotype from a background of nonmorphogenic cells and tissue. An outstanding illustration of this principle has been in somatic embryogenesis of cereals, in which a light-coloured, nodular callus with embryogenic competency can be isolated and maintained independently (Nabors et al. 1983).

Recent reports of somatic embryogenesis in conifers suggests that the capacity for embryogenesis is similarly confined to an unique phenotype that can be described as a white to translucent, mucilaginous callus (Hakman et al. 1985, Nagmani and Bonga 1985). Although these physical features may be sufficient to identify embryogenic conifer callus, they provide little insight into the critical features associated with or necessary for somatic embryogenesis in conifers.

To gain further insight into the process of somatic embryogenesis in conifers, biochemical and ultrastructural analysis of embryogenic and nonembryogenic calli were undertaken. To help ensure that actual physiological differences in the two tissue types were being measured, we took advantage of the observation that the culture of immature embryos of Norway spruce, near the cotyledon development stage, produces a mixed culture comprised of embryogenic and nonembryogenic calli. By manual separation over several subculture intervals, homogeneous callus lines of both embryogenic and nonembryogenic tissue were established. Thus, biochemical comparisons were made on embryogenic and nonembryogenic tissue of the same genotype and age, cultured under the same conditions.

The biochemical features measured were ethylene evolution rate, specific (glutathione) and total reductant concentration, and protein synthesis rate. Ethylene evolution rate and glutathione concentration were chosen because low levels of these compounds had previously been shown to be associated with somatic embryogenesis in wild carrot (Noland et al. 1985, Earnshaw and Johnson 1985). However, all the biochemical features measured fit several important criteria for use as indicators of an embryogenic (or nonembryogenic) state in conifer calli. Differences in the measured substances were of sufficient magnitude to unambiguously define the embryogenic competency of the tissue; the measurement could be done easily and rapidly on small quantities (<100 mg) of tissue. The measurement of ethylene evolution rate held the additional attraction of being nondestructive, enabling the reuse of the tissue in other experiments.

Biochemical analysis of the two tissue types revealed that, compared to embryogenic callus, nonembryogenic callus of Norway spruce evolved 20-100 times more ethylene, contained 5-7 times more GSH, reduced 20 times the amount of ferric ion, and synthesized protein at a rate 10-20 times slower.

At the present time it is difficult to assess the meaning of these results, although this does not preclude the use of these features as biochemical markers of embryogenesis. Ethylene has been found to have both inhibitory and stimulatory effects on morphogenesis. However, the ethylene evolution rate of nonembryogenic spruce callus approaches that of ripening fruit (which does not imply a desirable situation).

The elevated levels of GSH present in nonembryogenic tissue are consistent with previous observations that unorganized growth is associated with a high level of GSH. Embryogenic spruce callus presumably contains less GSH because it contains numerous differentiated structures (i.e., somatic embryos; 1,000

embryos/gm fresh weight) under the conditions examined. A similar situation in GSH levels has been reported in wild carrot somatic embryogenesis (Earnshaw and Johnson 1985). In carrot, elevated GSH levels were associated with proliferative growth (plus 2,4-D), whereas GSH levels dropped as embryo development proceeded in the absence of 2,4 D.

The enhanced capacity of nonembryogenic callus to reduce the ferric ion implies this tissue type possesses more reducing agents than embryogenic tissue. However, specificity tests to ascertain the nature of the reductants suggest that phenolic acids may be responsible for the reduction of ferric ion. These substances do not function as reducing agents *per se* but represent products of secondary metabolism. Therefore, it might be said that for nonembryogenic callus a cytological or chemical pattern of differentiation is the only morphological pathway available.

The particular biochemical analysis described was chosen in part due to its simplicity. The facile nature of these analyses enables them to be easily integrated into routine culture evaluation, adding a biochemical dimension to the assessment of morphogenic competency. However, it should be readily appreciated that markers of embryogenic potential are of limited value in successfully recognizing embryogenic conifer tissue. Alternative applications of these biochemical characteristics may be more useful. Before alternative applications can be found, it is necessary to determine whether the biochemical features measured are involved in deterministic or developmental events in embryogenesis.

To investigate this last issue enzymatic inhibition of the synthesis some of the measured biochemicals was performed during embryo differentiation (plus 2,4-D) or embryo development (minus 2,4-D). The enzyme systems inhibited were PAL (through the specific inhibitor aminooxyphenyl propionic acid (AOPP)) and glutamylcysteine synthetase (through the specific inhibitor buthione sulfoximine (BSO)). The affect of inhibiting these enzymes should be a reduction in the level of phenolic acids (with AOPP) and a decreased level of GSH (with BSO). In line with the biochemical characteristics of embryogenic callus, the dimunition of activity in both these enzyme systems should promote some aspect of embryogenesis.

The result showed that, rather than an enhancement of embryo formation, the addition of the above inhibitors promoted embryo development or maturation. Therefore, it might be reasonable to expect that perturbation of the biochemical systems examined will assist in the critical transition between embryo proliferation and embryo development. This is currently an important problem area because only a small percentage (~1%) of the differentiated embryos ever develop into plants. Higher frequencies of embryo conversion will be required to successfully exploit this system for plant propagation.

One obvious difference between the two callus phenotypes was colour. Although grown in the light, embryogenic spruce callus was colourless, whereas nonembryogenic callus was green. Not unexpectedly, nonembryogenic callus contained many times the amount of chlorophyll found in embryogenic callus and the chloroplasts present in nonembryogenic callus were similar in structure to those found in seedlings. In contrast, embryogenic callus contained plastids that can be identified as chloroplasts, but they lacked the internal thylakoid membrane organization of typical grana. A similar type of chloroplast has been observed in embryogenic callus of European larch, suggesting that these distinct plastids may be ultrastructural markers of an embryogenic state in conifers. Analysis of the chloroplasts in dark-germinated seedlings of spruce and larch revealed that these unusual chloroplasts are not an intermediate type in normal chloroplast development in transfer from dark to light conditions. Preliminary observations suggest that these entities may be embryo-specific, providing a definitive marker for the presence of conifer somatic embryos.

In conclusion, it appears that many of the biochemical aspects of somatic embryogenesis in angiosperms are directly applicable to gymnosperms. Although not unexpected, the similarity is somewhat surprising given the wide differences in physical features of the tissue culture systems used to manipulate gymnosperms. Nevertheless, factors influencing somatic embryogenesis in angiosperms can likely be exploited to achieve better control of the same process in gymnosperms.

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MOLECULAR ASPECTS OF PLANT DEVELOPMENT WITH SPECIAL APPLICATION TO CONIFERS

E.G. Kirby

Department of Botany
Rutgers University
Newark, NJ 07102
USA

Plant regeneration from a variety of explant sources has been reported for a number of conifers (Thorpe and Biondi 1984), and tissue culture regeneration of elite genotypes is currently being used for establishment of clonal seed orchards (Farnum et al. 1983). However, the application of plant biotechnology techniques in conifers awaits regeneration of whole plants with predictable performance from cell and callus cultures.

The interaction of auxins and cytokinins in the reproductive physiology of adventitious shoots in cotyledon cultures of Douglas-fir *Pseudotsuga menziesii* Mirb. (Franco) is well established (Cheng 1975). Adventitious shoots are produced with high frequency in cultured Douglas-fir cotyledons. This has enabled preliminary studies of biochemical events associated with morphogenesis (Hasegawa et al. 1979, Yasuda et al. 1980). Only an understanding of basic events during morphogenesis in regenerating systems (eg. cotyledons) will enable us to control events in cell and callus cultures of important conifers.

Histochemical analysis of early shoot-forming regions in tobacco tissue cultures has demonstrated that levels of RNA and protein increase while DNA levels remain constant, suggesting that increases in transcription and translation accompany shoot formation (Thorpe and Murashige 1970). Additional evidence for an altered metabolism in early shoot-forming tissues of tobacco is seen in the increased levels of enzymes associated with glycolysis, suggesting higher rates of respiration (Thorpe and Laishley 1973). Data of Hasegawa et al. (1979) support the model of altered protein metabolism associated with organogenesis in cotyledon cultures of Douglas-fir.

Cytokinins have been shown to alter RNA and protein synthesis within the first 24 h of culture in cotyledons of *Pinus radiata* (Villalobos et al. 1984). The specific nature of the RNA population was not determined. Cytokinins have been shown to specifically alter gene expression in a number of plant systems. Pumpkin cotyledons respond to cytokinins by exhibiting enhanced total protein synthesis and by increased levels of cycloheximide-sensitive hydroxypyruvate reductase activity (Chen and Leisner 1985). In addition, feminized male buds of *Mecurialis annua* yield cytokinin-induced organ specific RNA (Delaique et al. 1984).

Molecular events associated with auxin responses in pea stems have been examined by Theologis and Ray (1982). These investigators isolated poly(A)$^+$RNA and utilized an *in vitro* translation system to demonstrate that short-term treatment with auxin induced translation of five specific peptides. More recent work (Walker et al. 1985) has shown that adding cytokinin to elongating pea stems does not alter auxin-induced changes, suggesting that auxin and cytokinin function independently of one another in activating transcription. High-frequency bud induction in Douglas-fir cotyledons (induced by high cytokinin, low auxin) shows an increase in synthesis of low molecular weight proteins (16 000 and 20 000 daltons) as early as after two days (Hasegawa et al. 1979).

Although somatic embryogenesis is not directly analogous to caulogenesis, studies of carrot (Sung and Okimoto 1981) have demonstrated that the embryogenic response is associated with phytohormone control of translation. Transfer of carrot cells to an auxin-free medium enhanced production of specific proteins, termed E1 and E2. Production of these proteins has been used as an early marker for the embryogenic response. Additional work (Sung and Okimoto 1983) has shown that callus-specific and embryo-specific proteins are both regulated by auxin, but in an inverse fashion: auxin presence induces callus-specific proteins, whereas withdrawal from auxin induces embryo-specific proteins.

The picture emerging from recent work is that alterations of transcription and translation (i.e. gene expression) are rigidly controlled during plant development. During embryogenesis in cotton, seed storage protein

subfamilies are induced over time at the same relative rates (Dure et al. 1984). Although each subfamily is not produced at the same rate, increases in all subfamilies remained proportional to each other over the developmental sequence. This suggests that a specific developmental signal controls expression of all storage protein subfamilies.

The incorporation of labelled amino acids into proteins by Douglas-fir cotyledons, two days after culture on bud induction medium (Hasegawa et al. 1979), suggests that early transcripts should be present prior to 48 h. Early molecular events associated with auxin responses in pea stem tissue showed the appearance of specific poly(A)$^+$RNA as early as after 2 h in culture (Theologis and Ray 1982). These results suggest examination of early molecular events associated with caulogenesis in Douglas-fir cotyledons should begin as early as after 2 h culture on bud induction medium.

Using the guanidinium/cesium chloride method, we have isolated total RNA from the Douglas-fir cotyledons incubated for 4 h on liquid medium containing 5 M N_6-benzylaminopurine (BAP) and 5 nM naphthalene acetic acid (NAA). Controls consisted of treatments with 5 M BAP alone, 5 nM NAA alone, and no growth regulators. *In vitro* translation (wheat germ system) of poly(A)$^+$-selected RNA populations has shown that RNA from all treatments directed protein synthesis above endogenous levels. Analysis of the radiolabelled translation products was accomplished using one and two dimensional gel electrophoresis, followed by autoradiography. A low molecular weight protein (ca. 14 000 d) is induced by 5 nM NAA alone and by 5 nM NAA in combination with 5 M BAP. Our evidence is in agreement with results presented by Hasegawa et al. (1979) on incorporation of radiolabelled amino acids into differentiating cotyledons of Douglas-fir. However, the earlier work did not show that low molecular weight proteins were associated with tissues treated with auxin alone. Currently, we are examining temporal patterns for production of RNAs associated with specific growth regulator treatments and analysis on two dimensional gels of the translation products. Recent results indicate differences in many polypeptides, associated with both culture period and treatment with specific growth regulators. We will undertake the construction of a cDNA library to bud induction in Douglas-fir cotyledons followed by rigorous +/- screening of the library. This approach will allow us to establish clones which are associated with early transcriptional events in bud formation.

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DNA TRANSFER IN PINES

R. Sederoff[1], A.-M. Stomp[2], W.S. Chilton[3], and L.W. Moore[4]

[1]USDA Forest Service, P.O. Box 245, Berkeley, CA 94701, USA
[2]Dept. Forestry, North Carolina State Univ., Raleigh, NC 27695, USA
[3]Dept. Botany, NC State Univ., Raleigh, NC 27695, USA
[4]Dept. Botany and Plant Pathology, Oregon State Univ., Corvallis, OR 97331, USA

Introduction

The long generation times of forest trees are a significant barrier to the genetic analysis of many commercially important species. This barrier could be circumvented by the development of a molecular genetics methodology for directly transferring specific genes into the species of choice. Using such a method, the introduction of new and directed genetic changes could take place independent of the sexual cycle of forest trees. Our approach has been to develop a DNA transfer system in pines, because of their potential for genetic engineering and their importance as a source of wood and fibre throughout the world.

Several DNA transfer systems have been developed for animals and higher plants. The most direct system, developed first with animal embryos, is DNA microinjection. This method has been successful in animal cells and in tobacco. An alternative is direct uptake of DNA into cells, used successfully in higher plants. However, the best developed system for DNA transfer in higher plants has involved the use of the soil microorganism *Agrobacterium tumefaciens* (Gordon 1988). This bacterium, which infects some conifers, has the ability to transfer DNA into the cells of susceptible host plants. Previous attempts to infect pines of several species have been unsuccessful (De-Cleen and DeLay 1976). We argue that the host specificity of *A. tumefaciens* might not have been adequately tested and that, if pines were susceptible, a DNA transfer system might be readily developed.

Methods and Results

We attempted to find a susceptible combination of host and pathogen by testing a wide range of bacterial strains, isolated from diverse sources, on seedlings of loblolly pine *Pinus taeda* L. In our first series of 19 strains, we found two that were capable of inducing tumor formation on seedlings or on germinants in culture (Sederoff et al. 1986). The frequency of gall formation was low and further evidence was needed to establish that these galls, in fact, resulted from the transfer and expression of bacterial genes in cells of loblolly pine. To do this we established the tumor tissue in culture and assayed for opines, known to be in cells transformed by *A. tumefaciens* (Sederoff et al. 1986).

Opines are unusual metabolic products produced in gall tissue transformed by *A. tumefaciens*. They can, therefore, serve as chemical markers for DNA transfer. The opines are usually conjugates of monosaccharides, Krebs cycle or glycolytic intermediates, and amino acids. More than a dozen different opines have been isolated and characterized. Because *A. tumefaciens* strains differ in the opines that are produced when they infect plant cells, these compounds can "fingerprint" transformations by specific *A. tumefaciens* strains.

We tested for the presence of opines using transformed sunflower callus. In this fashion, we identified the specific opines anticipated if our galls were due to transferred DNA. Extracts of gall-derived pine tissue were prepared and assayed by high voltage electrophoresis and opine specific straining.

Extracts of pine gall-derived callus, originally derived from a gall produced in a seedling inoculated with *A. tumefaciens* strain U3, were found to contain agropine. Similarly, nopaline was found in high amounts in tissue derived from galls induced in loblolly pine by strain M2/73. These results conformed to our sunflower data, and suggests that gall formation was the result of DNA transfer by *A. tumefaciens* (Sederoff et al. 1986).

Our results with loblolly pine made us wonder about the host range of strains U3 and M2/73 in *Pinus*. We, therefore, conducted a larger survey to determine if gall formation

could be obtained with other pine species. It was found that several additional pine species -- sugar pine *Pinus lambertiana* Dougl., Virginia pine *P. virginiana* Mill., Scots pine *P. sylvestris* L., Monterey pine *P. radiata* D. Don., slash pine *P. elliottii* Engelm., ponderosa pine *P. ponderosa* Laws. -- were susceptible to *A. tumefaciens*. However, the level of susceptibility varied greatly among pine species and among bacterial strains on a given species. Assays were carried out to verify that transfer and expression of genes for opine synthesis had taken place. We conclude that the pines are generally susceptible to infection by *A. tumefaciens*, but some strain specificity is apparent.

An important step in the development of a general method for DNA transfer is the ability to transfer and express selectable markers. One such marker is resistance to amino-glycoside antibiotics. These molecules are effective inhibitors of protein synthesis in both prokaryotic and eukaryotic cells. Bacterial resistance to these compounds has been found, caused by the enzymatic phosphorylation of the antibiotic. One of these enzymes, neomycin phosphotransferase II (NPT II), has been transferred and expressed in several higher plants and confers resistance to kanamycin and related antibiotics (Bevan et al. 1983). Several vectors have been constructed for use in *A. tumefaciens*, with a chimeric gene containing NPT-II under control of the promoter for nopaline synthase (nos). We used pEND4K, a plasmid that contains a ColE1 replication origin, a multiple cloning site with alpha complementation, a λ cos site, and a broad host range in addition to the chimeric nos-NPT-II gene (Klee et al. 1985).

The pEND4K vector was introduced into a virulent armed strain and inoculated into sugar pine shoots *in vitro*. The resulting galls were cultured as callus for three transfers on a medium containing kanamycin. The resulting tissue was shown to have the activity of NPT II using the assay system of Reiss et al. (1984).

Conclusions

A. tumefaciens provides a tractable system for transferring and expressing foreign genes in pine cells. The methods will probably be practicable for several different commercially important pine species and indicate a significant potential for genetic engineering in these forest tree species. The major barrier to the realization of this potential remains the regeneration of a pine from transformed cells or tissues.

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TRANSFORMING PLANT CELLS

M.P. Gordon

Department of Biochemistry
University of Washington
Seattle, WA 98195
USA

Agrobacterium tumefaciens creates an ecological niche for itself by transforming host plant cells. Its ability to transform cells is associated with the presence of large catabolic plasmids, which are tumor-inducing plasmids or pTi. The region of the plasmid that is transferred and incorporated into the nuclear DNA of plant cells, the T-DNA, is bounded by two 24 basepair (bp) direct repeats. The T-DNA contains genes for synthesizing indoleacetic acid, isopentenyl AMP, and a series of opines.

T-DNA genes are eucaryotic and their expression results in the uncontrolled growth of transformed plant cells and the utilization of host metabolites for opine synthesis. The pTi is catabolic and enables the transforming bacterium to utilize opines. An additional region of the pTi, the vir region, is procaryotic in nature and contains six transcriptional loci, vir A, B, G, C, D, and E. Insertion mutation of any one of these loci destroys the virulence of the plasmid. The vir loci have been virtually completely sequenced.

We now have clues concerning the functions of some of the genes. Vir A is expressed constitutively and the gene product has been found in the membrane fraction of *A. tumefaciens* (Winans et al. 1986). We postulate that the product of gene A, a transducing protein, reacts with products of wounded plant cells, such as acetosyringone or polyphenols, resulting in increased transcription and activation of vir G. Such behaviour is similar to that observed in a number of transducing proteins interacting with transcriptional activators (Winans et al. 1986). The vir G product is then postulated to switch on vir B, C, D, and E. The products coded by the first two open reading frames in vir D produce single-stranded nicks at identical positions in the 24 bp direct repeats which flank the T-DNA (Yanofsky et al. 1986). Other workers, on the other hand, report a double stranded cut (Kovkolikova-Nicola et al. 1985).

The details of the reactions by which processed T-DNA is transformed and incorporated into the plant genome are not yet understood. Vir C has an effect on host range. A wide host range strain, A 348, induces a hypersensitive life response in grapes, while vir C mutants of this strain can successfully transform grapes (Yanofsky et al. 1985). An additional vir-encoded trait which we call "supervir" extends the host range of *A. tumefaciens* to agriculturally important legumes such as alfalfa and soybeans (Kumari 1985) and enhances the transformation of poplar (Pythoud et al. 1987).

The basis for the action and functions of other vir genes is unknown. A number of chromosomal genes are known to be necessary for virulence and, indeed, the pTi will only function in the chromosomal background of *Agrobacterium* and the closely related organism *Rhizobium*. The chromosomal virulence genes, A and B (Douglas et al. 1982), are required for absorption to plant cells and are interchangeable with the *Rhizobium* nod A and B genes (Dylan et al. 1986). The Ros mutation switches on vir C and D (Close et al. 1985).

Transformation of leaf disks by *A. tumefaciens* appears to be the procedure of choice for genetically engineering plants provided, of course, that the cloned genes are available and that plants can be regenerated from leaf disks (Horsch et al. 1985). It is also possible to transform protoplasts or rapidly dividing suspension cultures of plant cells with *A. tumefaciens*, or to fuse protoplasts with bacterial spheroplasts (Hasezawa et al. 1981).

Free DNA can also be used to transform plant cells and has an advantage in that the genes in question need not be cloned. Various procedures have been used to increase the permeability of protoplasts to DNA, such as high pH, polyethylene glycol, heat shock, and electroporation. The relative contribution of each of these conditions to the overall efficiency of transformation seems to vary from one laboratory to another (Potkykus et al. 1985). In our laboratory, we have found that with tobacco protoplasts, the best results are obtained by electroporation using electronic switching. The other parameters of the system are 250 volts/4 mm cell and 200-400 μ farad capacitors. Details are in Bradshaw et al. (1987). Enhancement of the permeability of protoplasts to DNA has also

been reported with corn, so that the transformation of vitally important monocots now appears to be imminent (Fromm et al. 1985).

Free DNA can also be incorporated into cells by direct microinjection. In all of the above procedures there appears to be some scrambling of DNA, but there does not appear to be any difficulty in isolating tissue in which the foreign gene is expressed (Van Lijsebettens et al. 1985).

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GENE EXPRESSION IN PLANTS

T.J. Parsons

Department of Biochemistry
University of Washington
Seattle, WA 98195
USA

It appears that the signals which regulate gene expression in plants are widely conserved throughout the plant kingdom. These signals are able to function when artificially linked to the coding sequences of heterologous genes. Eventually, the useful application of genetic engineering will be greatly aided by an ability to regulate the expression of foreign genes in specific ways, i.e. by restricting the expression of a foreign gene to a particular tissue within the plant, and by expressing a foreign gene at particular times in the development of the plant or in response to external signals. As a step towards this goal, we are attempting to clone a gene from poplar (*Populus* spp.) trees which is induced specifically in response to wounding, and to use the *Agrobacterium tumefaciens* transformation system to determine the ability of the regulatory regions from this gene to direct the expression of foreign genes in a wound-inducible manner.

The expression of eucaryotic genes involves many complex biochemical steps, and the regulation of expression within the cell can occur at many different levels within the pathways of gene expression. A primary determinant of gene expression occurs at the transcriptional level, and it is our (limited) understanding of transcriptional control mechanisms which constitutes the basis for our ability to usefully genetically engineer plants. Control of gene expression also occurs at the level of processing and export of the primary RNA transcripts from the nucleus, the stability of the mRNA, the translation of the mRNA, and post-translational modification of the protein product. A better understanding of these processes will greatly enhance genetic engineering programs.

Eucaryotic genes have an upstream, or 5′, regulatory region ahead of the actual protein-coding segment of the gene. This region determines the initiation site for RNA polymerase, the primary transcriptional enzyme. Additionally, there are sequences behind, or 3′, to the coding sequences which signal the requisite polyadenylation of the RNA transcript. The transcriptional apparatus of procaryotes differs from eucaryotes such that, in order for a bacterial gene to be expressed in an eucaryotic cell, it is necessary to fuse the bacterial coding sequences to eucaryotic 5′ and 3′ regulatory sequences. The regulatory regions from *A. tumefaciens* T-DNA genes, or the cauliflower mosaic virus 35S gene, are routinely used to direct the constitutive expression of bacterial genes within plant cells.

In the last few years upstream transcriptional enhancer elements have been identified in many animal genes. These are usually small (50-100) basepair (bp) sequence elements, which are able to enhance transcription initiation regardless of their position and orientation relative to the gene. In addition, enhancers frequently retain their function when linked to foreign genes. Many enhancers have been seen to be tissue specific, or activated in response to various environmental stimuli. Only recently have enhancer elements been identified in plant genes, and these may emerge as powerful tools for specifying when, where, and to what extent a foreign gene is expressed within a plant.

Enhancer function in a plant gene is strongly demonstrated in a recent report (Simpson et al. 1986) on the light-inducible chlorophyl *a/b* binding (*Cab*) protein gene from wheat. A 247 bp upstream segment was shown to be sufficient to confer the normal light-inducibility and tissue specificity of the *Cab* gene to the bacterial neomycin phosphotransferase gene. Furthermore, conservation of enhancer function is seen across wide taxonomic lines; the wheat enhancer retained its activity in dicotyledonous tobacco plants. Significantly, when two *Cab* enhancer elements were placed in tandem, the extent of transcriptional activation was doubled. The 247 bp segment also displays a "silencer" activity, being alone able to suppress the transcription of a foreign gene in root cells. Hence, this small, manipulable sequence element can be used in conjunction with foreign genes to specify expression in particular tissues, under specific environmental conditions, and at specific levels of induction. As research continues, molecular biologists may reasonably expect to have access to many such specific con-

trol elements, expanding the range of potential genetic engineering accomplishments.

Work is progressing in our lab to isolate a wound-induced gene from poplars, with the aim of analyzing the regulatory regions of such a gene, and to test the gene's ability to direct the expression of foreign genes. A complementary goal of our work is to refine the application of recombinant DNA technology to deciduous trees. Poplars are an attractive tree species for molecular biological research due to their small genome size, their ease of tissue culture, high regenerative potential, and their susceptibility to transformation by *A. tumefaciens* (Parsons et al. 1986, Fillatti et al. 1987). We are currently using differential cDNA hybridization techniques to screen a cDNA library from wounded poplar plants for clones of mRNAs which are present only in wounded plants.

In several instances, the physiological elicitors of inducible plant defense genes have been identified and purified (e.g. Ryan et al. 1985, Nothnagel et al. 1983). The regulatory regions from a gene, induced by such elicitors, might be used to direct the expression of a foreign gene; the foreign gene could then be activated at will by the application of trace amounts of a chemical elicitor. Expression of a foreign gene at all times, in all plant tissues, would rarely be the ideal result of genetic engineering efforts, and in many cases the constitutive presence of a foreign gene product might prove detrimental to the engineered plant. It is, therefore, desirable to direct research toward developing means to control the expression of genes which are artificially introduced into the plant genome.

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THE DEVELOPMENT OF GLYPHOSATE-TOLERANT *Populus* PLANTS THROUGH EXPRESSION OF A MUTANT *aroA* GENE FROM *Salmonella typhimurium*

J.J. Fillatti[1], J. Sellmer[2], B. McCown[2], B. Haissig[3], L. Comai[1], and D. Remenschnieder[3]

[1]Calgene, Inc., 1920 Fifth St., Davis, CA 95616, USA
[2]University of Wisconsin, Madison, WI 53706, USA
[3]USDA Forest Service, Rhinelander, WI USA

Introduction

Ineffective weed control is a major factor limiting the establishment and management of short rotation *Populus* plantations (Akinymiju et al. 1982, Hansen and Netzer 1985). At present, weeds are contained largely through mechanical means and by limited applications of herbicides when trees are dormant. These methods, however, are expensive and only partially effective. A gene for glyphosate tolerance, developed at Calgene, offers an unique opportunity for novel control of weeds through the development of herbicide-tolerant *Populus* cultivars by recombinant DNA technology. We set out to develop a glyphosate-tolerant *Populus* tree, and in this paper we will describe the strategy employed to achieve this goal.

Isolation and characterization of the mutant *aroA* gene

The herbicide glyphosate inhibits 5-enolpyruvylshikimate (EPSP) synthase, an enzyme involved in the biosynthesis of aromatic amino acids in plants (Steinrücken and Amrhein 1980, Amrhein et al. 1980). Tolerance to glyphosate can be mediated by the overproduction of the target enzyme (Amrhein et al. 1983) or by the presence of an altered enzyme (Comai et al. 1983, Rogers et al. 1983). The objective of this work was to test whether the expression of a mutant bacterial gene coding for an altered EPSP synthase enzyme confers resistance to the herbicide glyphosate when introduced into poplar.

In *Salmonella typhimurium*, EPSP synthase is encoded by the *aroA* locus. Mutants resistant to the herbicide glyphosate were obtained by mutagenizing cultures of *S. typhimurium* with ethylmethanesulfonate and then screening for the ability to grow on medium containing the herbicide. A mutant *aroA* gene was cloned from the resistant cultures. Plasmids containing this sequence were shown to complement strains of *S. typhimurium* with a mutation at the *aroA* locus. Sequence analysis demonstrated that the mutant allele of the *aroA* locus had a single cytosine-thymine transition which resulted in the substitution of serine for proline. This single basepair substitution caused a decrease in the affinity of the enzyme for glyphosate without affecting the kinetic efficiency of the enzyme (Stalker et al. 1985).

In order to obtain expression of this gene in plants the mutant *aroA* structural gene was fused with the 5′ regulatory sequences of the mannopine synthase gene from *Agrobacterium tumefaciens* (Barker et al. 1983). The chimaeric *aroA* gene was then introduced into binary *Agrobacterium* vectors described below.

Plant transformation

Agrobacterium tumefaciens provides a natural gene transfer system for many dicotyledonous plants. During the natural infection cycle of *A. tumefaciens*, bacterial DNA (T-DNA) is integrated into the host plant chromosome, resulting in the production of tumors on plants (Chilton et al. 1980). The tumor inducing genes can be deleted and substituted by heterologous genes without affecting the ability of *A. tumefaciens* to transfer T-DNA to plants (DeGreve et al. 1982). These modified strains of *A. tumefaciens* can be incubated with protoplasts, cell suspension cultures, or explants. Transformed plants, lacking oncogenic symptoms, can be obtained.

Because transformation with *A. tumefaciens* involves the incubation of bacterial cells with plant tissue (co-cultivation) and subsequent regeneration of only selected transformed cells, an efficient regeneration system and an effective selectable marker are required. Our first objective was to develop a regeneration system for poplar using a hybrid clone, *Populus alba* x *Populus grandidentata* (NC5339). This clone was chosen because shoot cultures could be main-

tained *in vitro* and utilized for clonal propagation, providing a sterile source of explant material. After testing different media, shoots were regenerated from *Populus* NC5339 leaf explants on a medium containing Murashige and Skoog minimal salts, sucrose (3%), benzyladenine (BAP) (1 mg/L), zeatin (1 mg/L), and Phytoagar (0.6%) at pH 5.6. Shoots developed at the cut edges of leaf explants 20-30 days after planting. Between 1-3 shoots developed on the average from 35% of the leaf explants plated. During development of this system we found the media composition, the pre-culture, and subsequent handling of leaf explants affected the regeneration rate. If shoot cultures were maintained on media containing BAP prior to explanting, shoot regeneration was inhibited. *Populus* is also very sensitive to H_2O stress, and we found that leaf explants had to be cut in the presence of H_2O or shoots did not develop. In addition the size and age of the leaf explants also affected the rate of regeneration. Younger leaves cut into segments at least 2 cm^2 regenerated best.

When *A. tumefaciens* is used as a vector for introducing foreign genes into plant cells, it is convenient to engineer a dominant selectable marker into the T-DNA along with the gene of interest. A selectable marker allows direct selection of transformed tissue by conferring resistance to an antibiotic. The most commonly used marker for plant transformation is the bacterial gene coding for neomycin phosphotransferase (NPT II') (Bevan et al. 1983, Fraley et al. 1983, Herrera-Estrella et al. 1983). This gene, which confers resistance to the antibiotic kanamycin, has proved to be an effective marker in crop species such as tobacco (Horsch et al. 1985), tomato (McCormick et al. 1986, Fillatti et al., in press) and lettuce (Michelmore, pers. comm.). To test whether selection for kanamycin resistance was appropriate for *Populus* NC5339, we plated leaf explants onto MS1BZ medium containing 0,20, 30, 40, 60, and 100 mg/L kanamycin. Concentrations of 60 mg/L or greater completely inhibited callus growth and shoot initiation; therefore, we used kanamycin at 60 mg/L in our co-cultivation experiments.

With a regeneration system in place and a suitable marker for selection, we began the *Agrobacterium*/explant co-cultivation experiments. The strain of *A. tumefaciens*, C58/587/85, used in the co-cultivation experiments, is a binary oncogenic vector containing a wild type C58 plasmid (Hamilton and Fall 1971) which acts *in trans* to promote transfer of the mini-plasmid pPMG 587/85 (Fillatti et al. 1987). This mini-plasmid has T-DNA border sequences flanking the two NPT II' genes and the *aroA* gene (Comai et al. 1985). The expression of the NPT II' gene is promoted by regulatory sequences derived from the octopine synthetase gene of *A. tumefaciens* strain A6 (Barker et al. 1983). The expression of the other NPT II' gene and the *aroA* gene is regulated by sequences derived from the mannopine synthetase gene in *A. tumefaciens* strain A6 (Barker et al. 1983).

A modified leaf explant co-cultivation system was used to transform poplar. Leaf explants from *in vitro* grown *Populus* NC5339 plants were cut into ~2 cm^2 segments in H_2O and preincubated on tobacco feeder plates for 24 h under low light conditions. The tobacco feeder plates were prepared two days prior to use by pipetting 0.5 mL of tobacco suspension culture onto petri dishes (100 x 25 mm) containing 50 mL of Murashige minimal organics medium, supplemented with 2,4-D (0.1 mg/L), kinetin (1.0 mg/L), thiamine hydrochloride (0.9 mg/L), potassium acid phosphate (200 mg/L), and Difco Bacto agar (0.8%). After two days a disc of sterile filter paper (Whatman 3 mm) was placed on top of the tobacco cells. The leaf segments were incubated in a liquid broth culture of strain pCGN58/587/85 (2×10^8 bacteria per mL) for 30 min., blotted, and then replaced on the tobacco feeder plates for 48-72 h. Following the co-cultivation period, leaf segments were plated onto regeneration medium containing carbenicillin (500 mg/L) and kanamycin (60 mg/L). Thirty-eight of the 40 shoots, which developed and subsequently rooted on medium containing kanamycin, exhibited NPT II' enzyme activity as assayed by the protocol of Reiss et al. (1984). A Western blot analysis was conducted on the kanamycin-resistant *Populus* NC5339 plants to demonstrate the presence of the bacterial *aroA* protein. With bacterial EPSP synthase antiserum we could detect a polypeptide with the correct molecular weight. This polypeptide was absent in untransformed plants.

To test whether the introduced *aroA* gene conferred tolerance to the herbicide glyphosate, transformants derived from independent transformation events and untransformed control

plants were sprayed with the equivalent of 0.16 or 0.25 lbs/acre glyphosate or with H_2O as a control. Poplar plants producing the *aroA* protein exhibited tolerance to the herbicide treatment while control untransformed plants did not.

Conclusion

We have demonstrated that expression of the mutant *aroA* gene confers tolerance to the herbicide glyphosate in *Populus*. Through further modifications of the DNA sequences controlling expression and compartmentalization of the enzyme, we expect to obtain higher levels of tolerance. To our knowledge this work represents the first demonstration of introduction and expression of foreign genes into a forest tree species. Gene transfer technology will provide a new and powerful tool for improving forest tree species and a system for studying the regulation of gene expression. Furthermore, the availability of a glyphosate-tolerant *Populus* cultivars may have a great impact on the economics of commercial pulp production from *Populus* by allowing chemical control of weeds. This would substantially decrease losses due to weed competition during the establishment and subsequent cultivation of *Populus* plantations.

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